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Ecological and morphological features of *Amyloodinium ocellatum* occurrences in cultivated gilthead seabream *Sparus aurata* L.; a case study.

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Abstract

Understanding the patterns of occurrence of the ectoparasite *Amyloodinium ocellatum* and the conditions that result in its maintenance at non-dangerous levels for gilthead seabream *Sparus aurata* could be very useful, since outbreaks of heavy infestation by this parasitic dinoflagellate can cause severe mortality in temperate aquaculture. We have evaluated the interactions between *A. ocellatum* and related environmental variables for the first time. Biotic and abiotic parameters of water quality in production ponds from a temperate aquaculture (Sado Estuary, Portugal) were monitored and subsequently analysed. Dissolved oxygen, water temperature, pH, phytoplankton biomass and salinity were closely related to *A. ocellatum* occurrences; dissolved oxygen, water temperature, pH and phytoplankton biomass had significant negative relationships with *A. ocellatum* trophonts, while salinity had a significant positive relationship with *A. ocellatum* trophonts in fish gills. Phytoplankton biomass was significantly correlated with increases of dissolved oxygen in production ponds. An increasing of rate of water renewal increased salinity, due to persistence of low water levels in production ponds during the water renewal procedure. Salinity negatively affected phytoplankton biomass and consequently the level of dissolved oxygen, raising the probability of *A. ocellatum* occurrences. Fish biomass in production ponds was correlated with the average and the maximum number of trophonts found in fish gills, highlighting the importance of defining stocking levels and production values in ponds. The present results help to improve understanding of the interactions between biotic and abiotic variables, fish farm management practices and parasite incidence in temperate terrestrial pond aquaculture.

A morphological feature of the *A. ocellatum* tomites cells in division phase, collected from the most infected fish gills, is discussed. We also give a description and illustration of the phases of the *A. ocellatum* life cycle.

Keywords: *Amyloodinium ocellatum*, environmental variables, life cycle, aquaculture

1. Introduction

Among the protozoan ectoparasites of fish, the most important is the peridinean dinoflagellate *Amyloodinium ocellatum* (Brown, 1931; Brown and Hovasse, 1946), an ectoparasite of marine and euryhaline warm water fish. It has been responsible for numerous and serious epidemics in mariculture throughout the world, in wild as well as in marine aquaria, causing severe epizootics (Brown, 1934; Lawler, 1979; Ghittino et al., 1980; Paperna, 1980; Baticados and Quinitio, 1984; Sindermann, 1990; Noga et al., 1991; Noga and Levy, 1995; Kuperman and Matey, 1999; Ramos and Oliveira, 2001; Cruz-Lacierda et al., 2004). *Amyloodinium ocellatum* is non-specific in its host selection, infecting a wide variety of fish (Lawler, 1980), and Colorni (1994) even refers to parasitism on the monogenean parasite of the fish gilthead seabream *Sparus aurata* L.

The parasite has a life cycle consisting of three stages. The dinospore, the infective stage, attaches to gills and skin of the host and loses its flagella to give rise to the trophont or feeding stage, which penetrates epithelial cells by means of its rhizoids (Lom and Lawer, 1973; Kuperman and Matey, 1999). After feeding, the trophont detaches from the host cell and falls to the bottom

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substrate where it forms a cyst wall to become the tomont, which is the reproductive stage and will produce the free-swimming infective dinospores.

Despite the importance of this organism in both wild and mariculture fish species, little is known about its ecology and the environmental conditions that favour its occurrences.

Environmental factors can strongly promote infestation of fish by external parasites (Khan and Thulin, 1991). In the Salton Sea, *A. ocellatum* infestation of young tilapias increased under unfavourable environmental conditions of the lake. The severity of fish infestation by *A. ocellatum* was determined by an interaction by the pathogen with abiotic variables, such as water temperature, salinity, oxygen concentration and nitrogen level. It has been suggested that the occurrence of *A. ocellatum* in fish gills is associated with specific variances in environmental variables such as temperature and salinity (Kuperman and Matey, 1999). However, the questions of whether and how occurrences *per se* of *A. ocellatum* in fish are correlated with environmental and biological variables are still unanswered. Thus, it is important to determine how its occurrences in fish gills relate to the variation of environmental and biological variables.

In general, it is not easy to illustrate the interactions between ichthyo-parasites like *A. ocellatum* and environmental and biological variables. When a process or an organism in nature is controlled by a set of interrelated variables, focused statistical searches may be the only way to understand the relative importance of different variables in the natural system (Lau and Lane, 2002; Bernard et al., 2004).

In Portugal, several outbreaks of *A. ocellatum* have been reported in aquacultures from the central west, southwest and southern regions (Menezes, 1994), where the main fish species in production are gilthead seabream and seabass (*Dicentrarchus labrax* L.). Fish are usually introduced into production ponds in April, with a weight ranging between 4-8 g wet weight.fish⁻¹, and captured in the following year, during summer. The growing period of gilthead seabream takes about 15 months. Infestations of the gilthead seabream by the gill parasite *A. ocellatum* may occur, particularly during summer. At the end of the growing season, when the water temperature increases within production ponds, the proliferation of the ectoparasite and the damage done to the developing fish can rapidly reach devastating proportions in terms of fish production (Noga and Levy, 1995).

The main objective of this work was to disentangle the interactions of some factors in relation to *A. ocellatum* occurrences in cultivated gilthead seabream gills and environmental and biological variables and fish farm procedures. For the first time, principal component and GLM regression analyses were applied in order to verify whether occurrences in fish gills by this parasite correlate with different environmental and biological variables and management practices in the terrestrial ponds of a semi-intensive fish farm, located in the central region of Portugal.

An attempt to study the surface structure of the dinospores was made to determine whether it could be a different species or strain from that described by Landsberg et al. (1994). However, it was only possible to detect, with scanning electron microscopy (SEM) observations, different tomont phases. An undescribed morphological feature of the cells observed by SEM in the tomont phase is discussed and descriptions and illustrations of the phases of the parasite life cycle are presented, based on SEM and light microscope observations.

2. Material and methods

2.1. Study site

This study was conducted on a marine fish farm located at Sado Estuary, southeast of Lisbon (Portugal) (38° 32' 19.39"N, 8° 45' 49.50"W). The fish farm is characterized by several land production ponds dug in the natural sediment with dimensions between 6000 and 12000 m². It is an open system, collecting water directly from the estuary, which circulates among all production ponds and then goes to a decantation area before release back into the estuary. This fish farm procedure is known as 'water renewal' and is tide dependent.

At the end of the fish growing period, the system generates a fish biomass of approximately 1 kg.m^{-3} , with an average weight of $320\text{-}350 \text{ g.fish}^{-1}$ that corresponds to the market size.

2.2. Sampling

Amyloodinium ocellatum monitoring was carried out in 2007 through the final 5 months of gilthead seabream growth until it reached the market size. A total of 250 specimens were caught and examined from 16 production ponds. From each production pond, a sample of five fish was used to quantify the number of *A. ocellatum* trophonts. The trophonts were counted from the 2nd left fish gill using a light microscope at 100x magnification.

Water quality parameters, dissolved oxygen (DO), water temperature (TEMP), salinity (SAL), pH (PH), total suspended solids (TSS) and phytoplankton biomass (PHYTO), were measured in all 16 production ponds at the same time as fish sampling. Dissolved oxygen and water temperature were measured with a portable oximeter (Hach HQ 40). Phytoplankton was collected at 30 cm depth using a sampling bottle, from which 500 ml of pond saltwater were filtered through a $25 \mu\text{m}$ mesh filter, cells concentrated in 20 ml sterile saltwater and fixed with lugol. Counts were made using a Sedgwick Rafter S50 cell counter and microphytoplankton expressed in cells.ml^{-1} .

Another set of monitored variables corresponded to the rate of water renewal (RE), a management practice, in production ponds, water temperature (TEMP7) and dissolved oxygen (DO7) for the last 7 days before the day of fish sampling. RE was defined as the number of times the water was renewed in the 7 days prior to fish sampling. DO7 and TEMP7 were always measured whether or not water renewal in production ponds was carried out. Since water renewal was done using the day-time high tide, DO7 and TEMP7 were always obtained 2-3 h before sunset.

Fish biomass expressed in kg.m^{-3} is the number of fish per production pond multiplied by the fish average weight and divided by the water volume of the pond.

Tomonts were collected from the most infected gills. Gills were washed with sterilized saltwater using a $75 \mu\text{m}$ filter and the tomonts were concentrated in a Petri dish and kept at 22°C . Tomont divisions were observed for 5 days.

2.3. Scanning electron microscope (SEM) observations

The specimens collected from the infected gills were killed and fixed in 2% glutaraldehyde solution buffered with 0.1 M sodium cacodylate buffer at pH 7.2 for 48 h, at 5°C . Post-fixation was with 2% osmium tetroxide solution for 12 h at 5°C . Specimens were dehydrated in a graded series of ethanol, critical point dried with liquid CO_2 , mounted on double sided adhesive tape on SEM stubs, coated with gold, viewed and photographed using a JEOL JSM-5400 SEM operating at 20kV accelerating voltage (Landsberg et al., 1994).

2.4. Data analyses

The occurrence of *A. ocellatum* trophonts in fish gills was explored using multivariate statistical approaches. Factor analysis and multiple linear stepwise regression analysis were employed to examine possible relationships between environmental, biological and management practices with *A. ocellatum* occurrences. Data were log-transformed.

Factor analysis helps to identify a smaller set of uncorrelated variables that account for a large proportion of the total variance in the original variables (Ludwig and Reynolds, 1988).

The two most significant factor axes were plotted and, in order to analyse relationships between environmental, biological and RE variables and *A. ocellatum* occurrences, a backward multiple regression analysis was employed. *Amyloodinium ocellatum* occurrence, obtained by logarithmic transformation, was the dependent variable, whilst factors obtained by factor analysis were the independent variables. Since factors were orthogonal, co-linear problems were avoided in regression analysis. Statistic analyses were conducted using Statistica software (version 6.0).

GLM simple regression analyses were performed to analyse the main origin of dissolved oxygen in production ponds and the effect of RE in the two sets of environmental and biological variables.

The origin of dissolved oxygen in production ponds was analysed using data from fish sampling day. PHYTO was considered the independent variable and DO, PH and TSS the dependent variables.

Regular water renewal in production ponds was carried out. Changes in physical and chemical conditions of the water ponds due to the renewal process, which is done as function of daily tides, can have marked effects on several variables. The effect of RE on environmental and biological parameters was analysed in two ways: a short-term period of 1 day, RE as the independent variable and DO7 and TEMP7 as the dependent variables. During the 7 days before fish sampling, dissolved oxygen and water temperature values were obtained daily. Since these daily values compose the mean values of DO7 and TEMP7, it was possible to verify the immediate effect of RE on DO7 and TEMP7; a medium-term period of 7 days, RE as the independent variable and DO, TEMP, PH, PHYTO, SAL and TSS as the dependent variables.

The Mann-Whitney non-parametric test was used to compare environmental and biological variables between the production ponds with fish carrying less than or more than ten *A. ocellatum* trophonts on their gills. Only the variables considered in factor analysis as factor 1 (F1) and factor 2 (F2) were used.

Non-linear regressions were used to evaluate the relationship between fish biomass in production ponds and the average and maximum number of trophonts found in fish gills.

3. Results

3.1. Relationships between *A. ocellatum* trophonts and environmental, biological and RE variables

In Table 1 we present the mean values for all variables per sampled production pond considered in this study. Table 2 presents the factor loading matrix and Fig. 1 shows the plot of the first two factors. The eigenvalues of the factors 1, 2 and 3 are 2.6, 1.8 and 1.1 (Table 2). F1 accounted for 36.9% of the total variance and was related to TEMP, DO and PH. F2 accounted for 25.3% of the total variance and was related to SAL and PHYTO. TSS was correlated with F3, explaining 15.8% of the total variance. Figure 1 shows the plot of the first two factors, which together account for 62.2% of the variance in the data. To examine the relative role of the three factors in the occurrence of *A. ocellatum* trophonts in gilthead seabream, a stepwise multiple regression analyses was performed:

$$\text{Trophonts} = 2.479 - 0.477 \times F1 + 0.246 \times F2 - 0.438 \times F3 \text{ (adj. } R^2 = 0.151, n = 169).$$

The multiple regression showed that all three factors were significantly associated with *A. ocellatum* occurrences. According to the signs of factor scores in the multiple regression and the associated variables (Table 2), trophont occurrences were negatively associated with DO, TEMP, PH and TSS. On the other hand, F2 was positively correlated with *A. ocellatum* occurrences. F2 included PHYTO and SAL, which presented an opposite trend (Table 2). This suggests that SAL is positively related with trophont occurrences and negatively related to PHYTO.

3.2. Variable differences within different number of *A. ocellatum* trophonts in fish gills

Mean values for variables related to F1 and F2 are presented in Table 3 for fish with less than or more than 10 *A. ocellatum* trophonts/gill. Variable values obtained from ponds with less than 10 *A. ocellatum* trophonts in fish gills seem to represent conditions that minimize parasite occurrence.

3.3. The origin of dissolved oxygen in production ponds

The variables DO, PH and TSS were positively associated with PHYTO, which is shown by the following regression equations:

$$\text{DO} = 4.27 + 0.09 \times \text{PHYTO} \quad (r = 0.50, \text{adj. } r^2 = 0.217, n = 49).$$

$$\text{PH} = 2.03 + 0.01 \times \text{PHYTO} \quad (r = 0.24, \text{adj. } r^2 = 0.057, n = 49).$$

$$\text{TSS} = 5.22 + 0.04 \times \text{PHYTO} \quad (r = 0.22, \text{adj. } r^2 = 0.044, n = 49).$$

This indicates that PHYTO in the studied period has influenced the values of DO, PH and TSS.

3.4. The rate of water renewal effect in production ponds

3.4.1. Effect of RE on DO7 and TEMP7 (short-term influence - 1 day)

RE was negatively correlated with DO7 and positively correlated with TEMP7, as shown by regression analyses:

$$\text{DO7} = 4.69 - 0.09 \times \text{RE} \quad (r = -0.18, \text{adj. } r^2 = 0.031, n = 50).$$

$$\text{TEMP7} = 3.19 + 0.06 \times \text{RE} \quad (r = 0.26, \text{adj. } r^2 = 0.067, n = 50).$$

This indicates that water renewal reduces DO7 with an unexpected increase in TEMP7.

3.4.2. Effect of RE on variables from fish sampling day (medium-term influence – 7 days)

RE seems to be linked to SAL as shown by the plot of factors (Fig. 1), indicating that water renewal increased SAL. A regression analysis confirms the positive relationship between them:

$$\text{SAL} = 3.61 + 0.04 \times \text{RE} \quad (r = 0.34, \text{adj. } r^2 = 0.110, n = 50).$$

A positive relationship was found between TEMP and RE, in a similar way to that found over the short-term scale for the effect of water renewal rate on TEMP7.

$$\text{TEMP} = 3.20 + 0.05 \times \text{RE} \quad (r = 0.19, \text{adj. } r^2 = 0.033, n = 50).$$

Negative relationships were found between RE and PHYTO and TSS. However, no medium-term effects were found between RE and DO and PH.

$$\text{PHYTO} = 4.12 - 1.18 \times \text{RE} \quad (r = -0.26, \text{adj. } r^2 = 0.067, n = 49).$$

$$\text{TSS} = 5.33 - 0.19 \times \text{RE} \quad (r = -0.22, \text{adj. } r^2 = 0.044, n = 50).$$

$$\text{DO} = 4.64 - 0.031 \times \text{RE} \quad (r = -0.04, \text{adj. } r^2 = -0.002, n = 50).$$

$$\text{PH} = 2.04 - 0.01 \times \text{RE} \quad (r = -0.08, \text{adj. } r^2 = 0.004, n = 50).$$

RE reduced PHYTO and TSS, shown by the negative relationships. In the medium-term effect, despite the fact that RE reduced PHYTO, no effects were seen on DO and PH values.

3.5. Fish biomass and *A. ocellatum* trophont occurrences

The average fish biomass in all production ponds was $1.00 \pm 0.45 \text{ kg.m}^{-3}$. The average weight and length of fish sampled was $274.1 \pm 92.0 \text{ g}$ and $233.2 \pm 26.0 \text{ mm}$, respectively. The

average and the maximum number of *A. ocellatum* trophonts/fish/production pond are presented in Table 3.

Non-linear relationships were found between fish biomass in the 16 production ponds and the average number of trophonts per fish (Fig. 2) and the maximum number of trophonts per fish.

Average number of trophonts = $-22.34 + e^{(3.04 + (0.79 \times \text{fish biomass}))}$ ($r = 0.59$, variance explained = 34.54%).

Maximum number of trophonts = $-429.15 + e^{(5.98 + (0.35 \times \text{fish biomass}))}$ ($r = 0.55$, variance explained = 30.33 %).

3.6. An undescribed feature of *A. ocellatum* tomont and parasite life cycle

No typical clinical signs and effects were observed of an *A. ocellatum* infection (decrease or lack of feeding activity, flashing and coughing). Fish captured with values of more than 300 parasites per gill apparently do not show these symptoms. However, after capture, fish with *A. ocellatum* trophonts were less active and died more quickly.

In our SEM studies, an orifice in some tomont division cells was detected after the 64-cell stage (Fig. 3A). A membrane partially covering the outside cells of the tomont was also detected (Fig. 3B). Dinospores were not detected in the SEM studies, but the dinospores observed with LM (Fig. 4.3) were similar to those described by Brown (1934).

LM observations showed that, after several divisions, individual tomont cells presented a rotation movement, resulting in what seems to be the dinospore.

The life cycle phases of *A. ocellatum* were observed using SEM and LM (Fig. 4).

4. Discussion

4.1. Ecological features of *A. ocellatum* occurrences in cultivated gilthead seabream

The present results indicate that the occurrence of *A. ocellatum* is negatively related to DO, TEMP, PH, PHYTO and TSS and positively related to SAL.

Despite the use of multivariate statistical analyses to explore relationships between *A. ocellatum* and environmental, biological and fish farm practice variables, the complexity of the system requires two approaches. One is to understand the interaction between DO, PH and PHYTO in terrestrial fish ponds. Paddle wheels aerators are the only device used to maintain the minimum oxygen levels for fish and cannot increase dissolved oxygen concentrations beyond 100%. Therefore, the observed dissolved oxygen values, which were higher than 100%, must be the result of enhanced primary productivity within production ponds. A positive relationship was observed between DO and PHYTO during this period. Oxygen production by phytoplankton exceeded the consumption by the fish and decomposing wastes. In the present study, diatoms were the most abundant group within the microphytoplankton size fraction. The enrichment of phytoplankton in production ponds is enhanced by adding pellets to feed fish. Fish excrete most of their excess metabolic nitrogen as unionized ammonia (NH₃) rather than urea and CO₂ (aq.) (Randall and Wright, 1987). In the aqueous phase, ammonia forms a weak acid where NH₃ is in equilibrium with the ammonium ion (NH₄⁺). As pH increases, carbonate increases and bicarbonate and molecular CO₂ decrease. At the average pH of seawater (pH 8.2), only about 1 % of total CO₂ is found as molecular CO₂, 90 % as HCO₃³⁻, and the rest as CO₃²⁻ (Nielsen, 1975). The excreted products seem to promote phytoplankton growth (Eyre, 2000) and, consequently, lead to increase of dissolved oxygen values and pH in accordance with our results. The interaction between ammonium and nitrate uptake by phytoplankton is a complex process (Dortch, 1990) and beyond the scope of this study. However, uptake of N-compounds by phytoplankton is well documented (Goering et al., 1964; Harvey and Caperon, 1976; Eppley et al., 1979; Goldman and Glibert, 1983; Glibert and

McCarthy, 1984). Uptake of inorganic carbon by phytoplankton during photosynthesis has the potential to increase pH in the surrounding water (ter Braak and van Dam, 1989; Dixit et al. 1992; Hansen, 2002), which is also consistent with our results.

The other approach is to understand the effects of pondwater renewal on environmental and biological variables. Although RE does not directly influence the occurrences of *A. ocellatum* in fish gills, as shown by the multivariate analyses, it had implications for the two sets of environmental variables DO7 and TEMP7 and data from the fish sampling day, DO, TEMP, PH, PHYTO, SAL and TSS. Changes in physical and chemical water conditions within production ponds, due to water renewal, can have marked effects on phytoplankton (Garget and Marra, 2002; Cermeño et al., 2006; Henríquez et al., 2007). In this study, those effects could explain the reduction in DO7 over the short-term scale of 1 day. Within the production ponds, phytoplankton is responsible for dissolved oxygen production but, when a part of the pondwater is removed, phytoplankton biomass will also decrease. It is important to note that the estuarine water used to replace the water from production ponds has lower dissolved oxygen values and lower chlorophyll a concentration than the water inside the ponds (Duarte and Henriques, 1991; Pereira, 2006). The short-term effect of RE in production ponds leads to a decrease DO7. However, an unexpected increase in water temperature was shown by the positive correlation with the variable TEMP7. A possible explanation for this result is that when water renewal was undertaken the production ponds had low water levels for at least 3-4 h with a mean depth of approximately 0.5 m; during this time they were exposed to the sun and high summer air temperatures.

In the medium-term effect, a negative relationship was found between RE and PHYTO and TSS. Despite RE resulting in a decrease in PHYTO, DO and PH are not affected in the medium-term. This could be due to water renewal being followed by a stabilization of the water strata, which would leave the remaining phytoplankton exposed to the photic zone long enough to make use of nutrients (Smetacek and Passov, 1999) and consequently increase its biomass. As a result, in the medium-term effect, dissolved oxygen balance and pH remained unchanged despite systematic fluctuations in phytoplankton biomass. The increase of water renewal frequency increases the total time for which pondwater is shallow, and, thus, contributes to increased water temperature and salinity. This is supported by the positive relationships between RE and TEMP and between RE and SAL, expressed by medium-term effects regressions. Factor analyses indicate a stronger relationship between RE and SAL compared with those between RE and TEMP (Fig. 1). Salinity stress strongly influences phytoplankton primary production (Jouenne et al., 2005), which is in agreement with the opposed signs obtained in factor analyses (Table 1, Factor 2). SAL is the only environmental variable positively correlated with *A. ocellatum* trophonts, having an important role in parasite occurrences that is also supported, in part, by Kuperman and Matey (1999), who pointed out that a combination of high water temperature and high salinity levels promoted heavy infestation by *A. ocellatum*. Despite TEMP increase with RE it seems not to affect *A. ocellatum* occurrence in fish gills, as shown by Table 2. No differences in TEMP were found between fish gills less than 10 trophonts /gill and more than 10 trophonts/ gill. A possible explanation is that continuous water renewal in production ponds during summer, with daily tides, will slowly increase salinity and, consequently, the frequency of *A. ocellatum* occurrences.

What are interesting and new are the results obtained by factor analyses, particularly where the major variables DO, TEMP and PH act in combination with PHYTO to decrease *A. ocellatum* trophont occurrences (Table 2, Factor 1). These data lead us to a different perspective for dealing with *A. ocellatum*, based on best management practices in aquaculture (Boyd, 2003). The new question is not why *A. ocellatum* occurs but how it can be avoided.

Production lakes are enclosed bodies of water that receive anthropogenic inputs and, like other enclosed bodies of water (lagoons, salt ponds, and embayments), can be exposed to conditions of high pH, high phytoplankton production and low oxygen levels (King, 1970; Oviatt et al., 1986; Chen et al., 1994). The studied production ponds presented reasonably high values of dissolved oxygen, which indicates that, despite phytoplankton fluctuations due to water renewal process, a reasonable pattern of water quality was maintained and that converting waste into algal biomass

rarely reached a eutrophic state (Shpigel and Fridman, 1990; Shpigel and Blaylock, 1991; Schwartz and Boyd, 1994; Neori and Shpigel, 1999; Neori et al., 2004).

Phytoplankton is important in the ecology of complex estuarine food webs. *Amyloodinium ocellatum* is one of the many organisms that compose estuarine food webs and its population dynamics should be controlled by the roles of predator-prey or consumer-resource relationships. Increased phytoplankton would cause the zooplankton to increase. Those microorganisms and bacteria could feed on *A. ocellatum* at some stage of its life history and thus lessen chances of increase in its numbers (Brown, 1934).

Production ponds with water conditions similar to those shown in the present results for fish sampled with less than 10 trophonts/gill (Table 2), imitate to a certain extent natural systems with complex food webs and the population dynamics found therein (Folke and Kautsky, 1992). These types of ponds are referred to as “green pond systems” (Neori et al., 2004), having a water quality pattern where the probability of *A. ocellatum* occurrences will be minimized.

Another important aspect in this “green pond system” is fish stocking. Our data shows a significant and positive relationship between density of fish and *A. ocellatum* occurrences, indicating that production ponds with higher values of fish stocking seems to be more prone to *A. ocellatum* occurrences. Higher values of fish stocking had necessarily higher feed inputs, which could change dramatically any unstable balances of the “green pond system”, causing severe variation in environmental and biological variables and an inevitable increase in probability of production losses due to a disease outbreak (Tveteras and Heshmati, 1998; Martinez-Cordero and Leung, 2004).

Finally, to maintain a “green pond system” two management practices for the second summer of gilthead seabream (the end of production) must be considered. The water renewal should be performed utilising night tides when dissolved oxygen from the target production pond becomes lower than 100%, measured 2-3 h before sunset. This could help to change physical and chemical water characteristics, promoting better pondwater conditions for phytoplankton development (Mangoni et al., 2008; Jouenne et al., 2005). On the other hand, excessive phytoplankton growth may occur, increasing dissolved oxygen values beyond 180% saturation. In such cases, water renewal should have the objective of reducing the phytoplankton biomass. Other important management practices are the selection of stocking ($1.02 \text{ kg} \pm 0.43 \text{ kg.m}^{-3}$) and feeding rates that should not exceed the assimilation capacity of the ponds (Neori et al., 2004; Boyd, 2003).

In the “green pond system”, with selected fish stocking density and water renewal with night tides, phytoplankton primary production acts as a regulator of oxygen saturation and as a sink for nutrients. This integrated system of production ponds mimics as far as possible the functioning of natural ecosystem (Folke and Kautsky, 1992), and is important in the maintenance of *A. ocellatum* at non-dangerous levels for fish.

4.2. Morphological features of *A. ocellatum* tomont in cultivated gilthead seabream

One of the morphological aspects of the tomont is the occurrence of an “eye-spot” first observed at the 4-cell stage and more easily at the 32-cell stage onwards, using LM (Brown, 1934). In this study, SEM observations revealed an orifice (Fig. 3A) at the 64-cell stage onwards, occurring only in some cells of the tomont (Fig. 3B). This is the most particular feature of the tomont phase, and could be the same as that observed by Brown. Brown (1934) could not analyse the three-dimensional surface of the tomont as can now be observed by SEM, so probably could only see the inner part of this orifice, presented as an elongated mark. Another morphological aspect is the presence of a membrane within which the tomonts are enclosed (Brown, 1934). However, the SEM studies showed that this membrane only partially covers the outer cells of the tomont (Fig. 3B).

The LM observations of the dinospores were more similar to those referred to by Brown (1934) than those by Landsberg et al. (1994). The observation of cell sporulation during this study gives the impression of a metamorphosis and not a sporulation as argued by Brown (1934).

A description and illustration of the phases of the *A. ocellatum* life cycle, based on SEM and LM photographs, is presented for the first time (Fig. 4).

5. Conclusions

Our results indicate that infestations of the gilthead seabream by the gill parasite *A. ocellatum* can be avoided if a defined pattern of water quality is kept within production ponds with a defined fish stocking level. This pattern of water quality can be achieved by water renewal with night tides, which should be carried out by considering the dissolved oxygen values in production ponds.

The morphological feature of *A. ocellatum* tomont phase, described by Brown (1931) as an “eye-spot”, is an orifice occurring in some cells.

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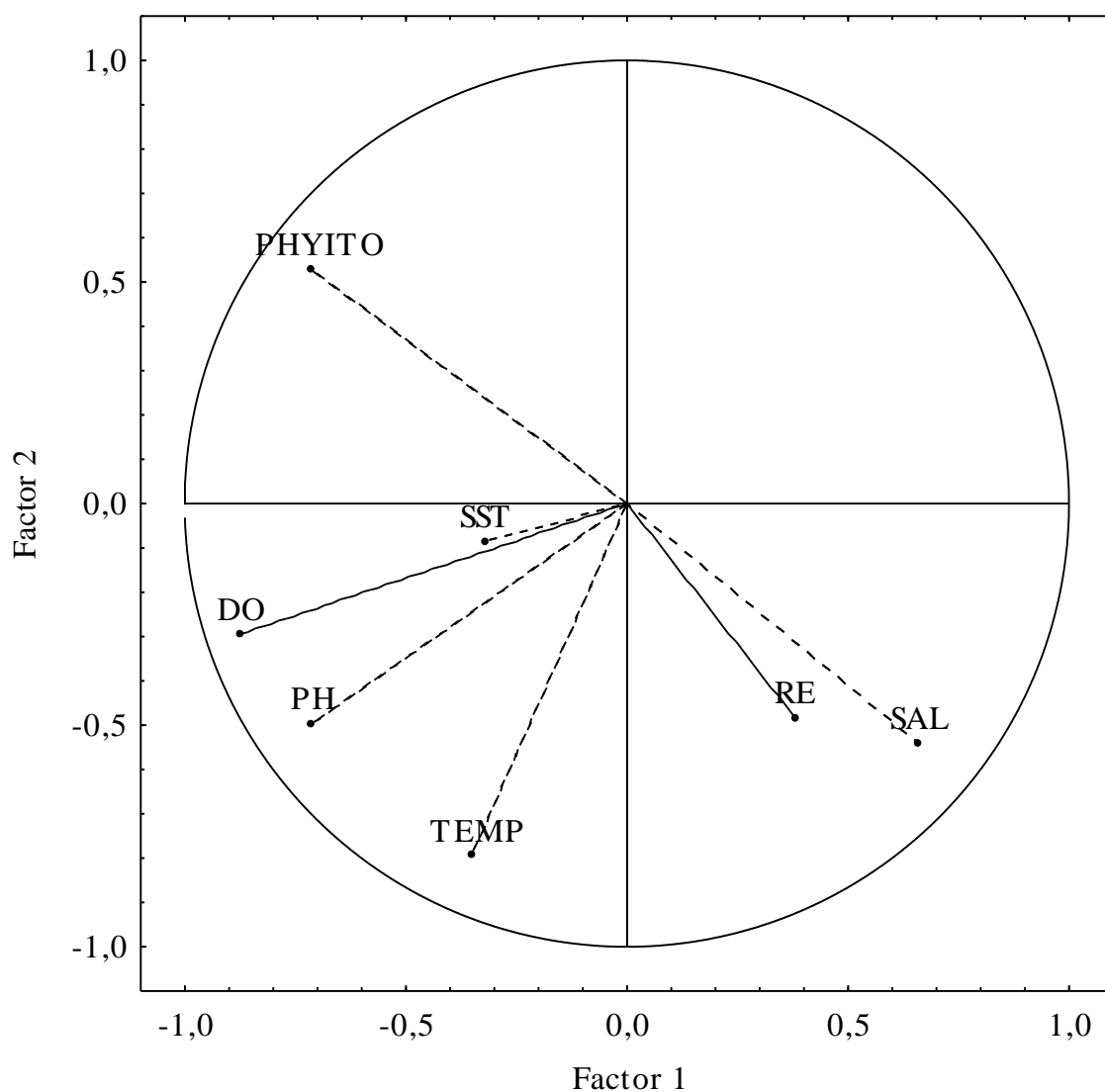


Fig.1. Projection of the environmental, biological and rate of water renewal variables on the factor plane (Factor 1 \times Factor 2).

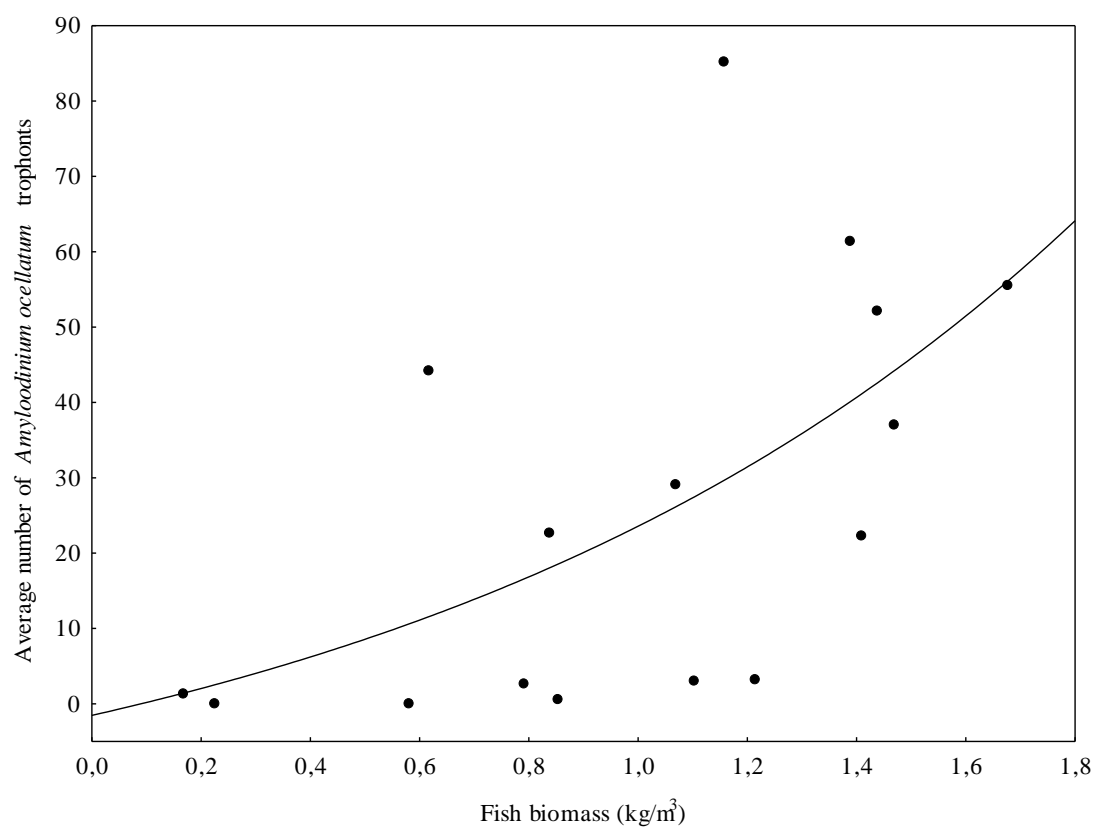


Fig.2. Non-linear correlation between average number of *Amyloodinium ocellatum* trophonts and fish biomass in production ponds.

Fig.3. Scanning electron microscope micrographs of a tomont cell with the characteristic orifice. A; 5000 \times . A tomont with the cell-membrane visible as referred to by Brown (1931) and some cells with orifices. B; 1500 \times .

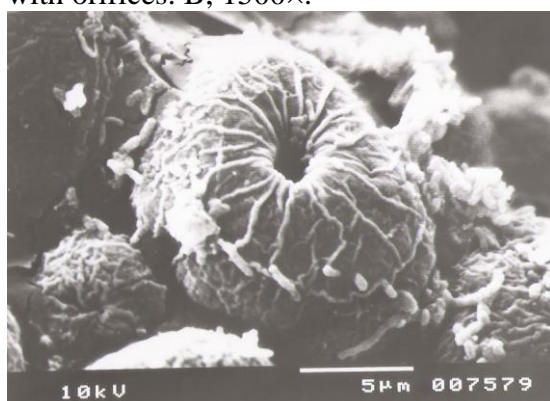


Fig. 3a

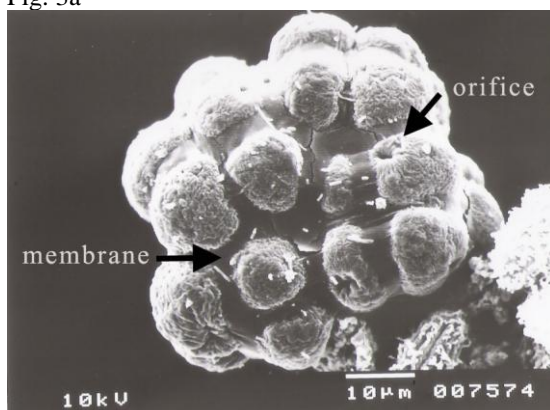


FIG. 3B

Fig.4. Life cycle phases of *Amyloodinium ocellatum*. A; Trophont attached to fish gills - drawing based on scanning electron microscope (SEM) microphotographs from Kuperman and Matey (1999). B; Tomont (4 cells). B1; Tomont (16 cells). B2; Tomont (128 cells) - all tomont drawings based on SEM microphotographs obtained in this study. C. Dinospore - drawing based on SEM microphotographs from Landsberg et al. (1994). 1 to 3. Light microscope photographs. 1 and 1a; Trophont attached to fish gills with apparent inflammation of epithelium. 1b; Trophont after detachment from fish gills, enclosed in a cell membrane. 2 to 2c; Tomont division phases. 3; Dinospores.

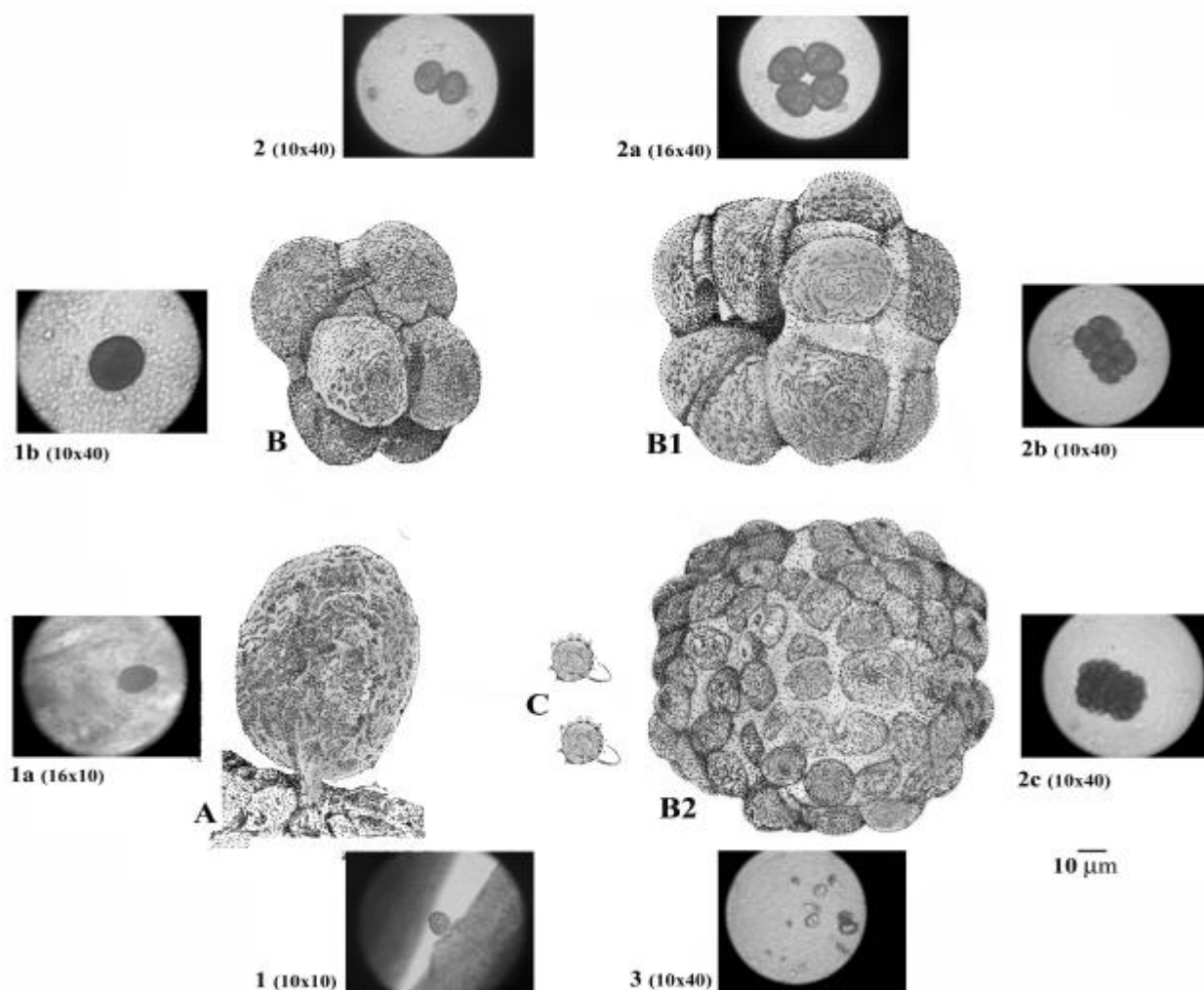


Table 1. Variables for the studied period/sampled production pond: Dissolved oxygen (DO), water temperature (TEMP), salinity (SAL), pH (PH), total suspended solids (TSS), phytoplankton biomass (PHYTO), mean and maximum number of *Amyloodinium ocellatum* trophonts/gill, fish biomass, rate of water pond renewal (RE) in the last 7 days, mean dissolved oxygen (DO7) and mean water temperature (TEMP7) in the last 7 days, obtained 2-3 h before sunset. Values are means \pm standard errors.

Lake	DO (%)	TEMP (°C)	SAL (‰)	PH	TSS (mg.l ⁻¹)	PHYTO (cells.ml ⁻¹)	<i>A. ocellatum</i> (max. trophonts; n° of sampled fish)	Fish biomass (kg.m ⁻³)	RE	DO7 (%)	TEMP7 (°C)
1	134.4 (1*)	24.5 (1)	35.0 (1)	7.7 (1)	290.0 (1)	-	0.4 \pm 0.2 (1;5)	0.85 \pm 0.2 (5)	0.63 (1)	110.2 (1)	23.1 (1)
2	110.1 \pm 1.1 (2)	22.9 \pm 0.4 (2)	35.0 (1)	7.7 \pm 0.0 (2)	290.5 \pm 23.4 (2)	1567.0 \pm 96.4 (2; 7**)	2.5 \pm 0.7 (7;10)	0.79 \pm 0.1 (10)	0.50 \pm 0.0 (2)	122.2 \pm 5.8 (2)	23.1 \pm 0.3 (2)
3	166.6 \pm 13.1	25.8 \pm 0.8	35.0 (2)	8.1 \pm	436.0 \pm 26.3	1481.0 \pm 18.9	3.1 \pm 1.3 (12;10)	1.22 \pm 0.1 (10)	0.47 \pm 0.0	114.7 \pm 0.6	22.2 \pm 0.5

	(2)	(2)		0.0 (2)	(2)	(2; 14)			(2)	(2)	(2)
4	95.1 ± 3.7 (4)	22.6 ± 0.3 (4)	34.8 ± 0.1 (4)	7.6 ± 0.0 (4)	244.8 ± 7.8 (4)	301.5 ± 13.4 (4; 28)	85.2 ± 28.1 (436; 20)	1.16 ± 0.0 (20)	0.73 ± 0.0 (4)	101.2 ± 1.6 (4)	21.7 ± 0.2 (4)
5	98.3 ± 3.9 (7)	25.7 ± 0.2 (7)	37.3 ± 0.2 (7)	7.7 ± 0.0 (7)	264.6 ± 7.6 (7)	45.3 ± 5.2 (7; 49)	52.0 ± 16.3 (449; 35)	1.44 ± 0.1 (35)	0.63 ± 0.0 (7)	105.5 ± 2.2 (7)	24.9 ± 0.2 (7)
6	128.2 ± 12.7 (2)	25.3 ± 0.1 (2)	36.5 ± 0.4 (2)	7.7 ± 0.0 (2)	175.5 ± 16.5 (2)	84.0 ± 10.9 (2; 14)	61.4 ± 28.0 (269; 10)	1.39 ± 0.2 (10)	0.68 ± 0.0 (2)	96.6 ± 1.6 (2)	25.1 ± 0.0 (2)
7	87.3 ± 3.1 (7)	24.0 ± 0.2 (7)	36.1 ± 0.2 (7)	7.6 ± 0.0 (7)	179.6 ± 6.8 (7)	199.0 ± 39.3 (7; 49)	55.5 ± 9.7 (222; 35)	1.68 ± 0.1 (35)	0.74 ± 0.0 (7)	97.9 ± 1.4 (7)	23.7 ± 0.1 (7)
8	87.0 (1)	22.1 (1)	37.0 (1)	7.7 (1)	232.0 (1)	13.7 ± 3.0 (1; 7)	3.0 ± 0.7 (5; 5)	1.10 ± 0.0 (5)	0.56 (1)	159.8 (1)	24.0 (1)
9	94.0 ± 2.6 (3)	23.1 ± 0.5 (3)	36.0 ± 0.3 (3)	7.8 ± 0.0 (3)	279.3 ± 9.1 (3)	204.1 ± 54.3 (3; 21)	36.9 ± 9.3 (93; 15)	1.47 ± 0.1 (15)	0.40 ± 0.0 (3)	116.0 ± 3.2 (3)	22.5 ± 0.2 (3)
10	85.0 ± 0.7 (2)	21.2 ± 0.3 (2)	39.0 (2)	7.6 (2)	283.0 ± 7.5 (2)	19.6 ± 3.4 (2; 14)	44.2 ± 20.1 (183; 10)	0.62 ± 0.0 (10)	0.80 ± 0.0 (2)	102.6 ± 0.3 (2)	25.1 ± 0.2 (2)
11	107.1 (1)	19.2 (1)	35.0 (1)	7.9 (1)	135.0 (1)	688.0 ± 80.8 (1; 7)	1.2 ± 0.6 (3; 5)	0.17 ± 0.0 (5)	0.25 (1)	114.0 (1)	21.3 (1)
12	137.9 (1)	29.2 (1)	38.0 (1)	8.6 (1)	250.0 (1)	62.1 ± 9.7 (1; 7)	0.0 (0; 5)	0.58 ± 0.2 (5)	0.56 (1)	160.3 (1)	27.9 (1)
13	88.6 (1)	25.2 (1)	35.0 (1)	7.7 (1)	126.0 (1)	8.0 ± 2.4 (1; 7)	0.0 (0; 5)	0.23 ± 0.0 (5)	0.50 (1)	141.8 (1)	22.5 (1)
15	133.3 ± 4.2 (4)	26.3 ± 0.4 (4)	37.0 ± 0.3 (4)	8.0 ± 0.0 (4)	221.8 ± 6.6 (4)	467.6 ± 106.6 (4; 7)	22.7 ± 6.6 (88; 20)	0.84 ± 0.0 (20)	0.98 ± 0.0 (4)	125.2 ± 3.2 (4)	25.0 ± 0.4 (4)
16	142.4 ± 9.2 (6)	25.4 ± 0.2 (6)	36.0 ± 0.2 (6)	7.9 ± 0.0 (6)	212.8 ± 9.6 (6)	195.2 ± 28.0 (6; 42)	22.3 ± 6.8 (160; 30)	1.41 ± 0.1 (30)	0.89 ± 0.0 (6)	125.0 ± 3.6 (6)	24.0 ± 0.3 (6)
17	105.6 ± 3.7 (6)	23.6 ± 0.3 (6)	37.5 ± 0.3 (6)	7.7 ± 0.0 (6)	206.0 ± 2.2 (6)	144.6 ± 40.0 (6; 42)	29.1 ± 8.0 (210; 30)	1.07 ± 0.1 (30)	0.98 ± 0.0 (6)	112.1 ± 2.3 (6)	24.1 ± 0.2 (6)

* Number of samples/pond. ** Number of replicates.

Table 2. Factor loading matrix for *Amyloodinium ocellatum* trophonts related variables after a varimax rotation.

Variables	Factor 1	Factor 2	Factor 3
RE (rate of water renewal)	0.148	0.468	-0.543
TEMP (water temperature)	0.840*	0.232	-0.175
DO (dissolved oxygen)	0.814*	-0.442	0.096
SAL (salinity)	-0.087	0.924*	0.114
PH (pH)	0.827*	-0.127	0.254
TSS (total suspended solids)	0.179	0.082	0.891*
PHYTO (phytoplankton biomass)	0.097	-0.870*	0.174
Eigenvalue	2.6	1.8	1.1

* Significant values.

Table 3. Water variables from ponds with fish sampled for *Amyloodinium ocellatum* trophonts..

	TEMP ^a (°C)	DO ^a (%)	PH ^a	SAL ^b (‰)	PHYTO ^b (mg.l ⁻¹)
Less than 10 trophonts/gill	24.52 ± 0.23 (135)*	122.36 ± 3.47 (135)	7.83 ± 0.02 (135)	36.08 ± 0.14 (125)	447.32 ± 49.21 (129)
More than 10 trophonts/gill	24.24 ± 0.15 (115)	96.19 ± 2.52 (115)	7.66 ± 0.01 (115)	36.80 ± 0.15 (115)	113.48 ± 20.23 (106)
Mann-Whitney test	z = 1.19 p = 0.236	z = 6.35 p < 0.001	z = 5.90 p < 0.001	z = -3.42 p < 0.001	z = 4.40 p < 0.001

* Number of sampled fish gills. a - variables related to Factor 1; b - variables related to Factor 2.